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The HER2 proto-oncogene is amplified and overexpressed in approximately 25% of breast cancers. Amplification and overexpression of HER2 is correlated with poor patient prognosis, lack of responsiveness to tamoxifen treatment, responsiveness to adriamycin chemotherapy and responsiveness to Herceptin anti-HER2 immunotherapy. In this proposal we are characterizing changes in cell adhesion related to HER2 overexpression. We have investigated the effect of HER2 overexpression on alterations in cell adhesion in 3 cell lines engineered to overexpress HER2. Relative to parental control cell lines, HER2-overexpressing cell lines showed altered cell adhesion on selected extracellular matrix proteins, suggesting that integrin receptors are involved in this process.

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Introduction

The human epidermal growth factor receptor 2 (HER2) is amplified and overexpressed in 20-30% of breast cancers and is correlated with poor patient prognosis, lack of responsiveness to tamoxifen treatment, responsiveness to adriamycin chemotherapy and responsiveness to Herceptin (monoclonal anti-HER2 immunotherapy). Although amplification and overexpression of HER2 is important in the clinical management of breast cancer, relatively little is known about how overexpression of this gene leads to a more aggressive breast cancer with an increased occurrence of tumor cell metastasis. Our primary goal in this proposal was to characterize the molecular mechanisms contributing to alterations in adhesion in cells that overexpress HER2. Altered cell adhesion is an important characteristic of tumor cells because it is required for anchorage-independent growth, cell migration and cell invasion. Characterization of the molecular basis for alterations in tumor cell adhesion may provide a better understanding of the molecular mechanisms involved in more complex behaviors of tumor cells. An understanding of the mechanism(s) of altered cell adhesion may allow us to restore normal cell adhesion to cells that overexpress HER2.

Body

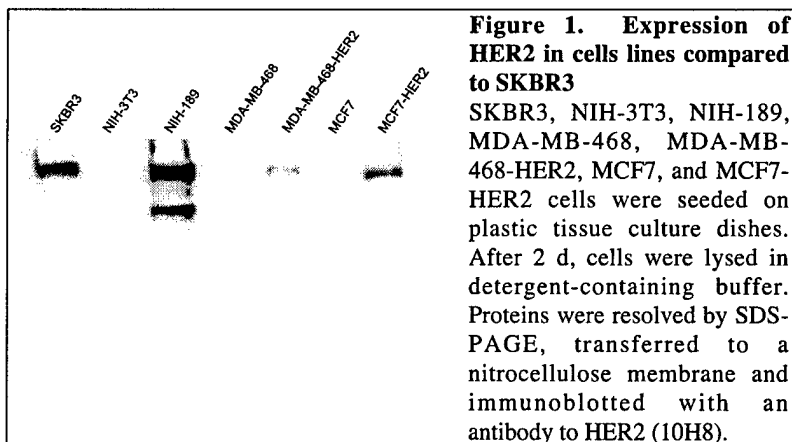
Although a number of studies have shown that HER2 overexpression causes transformation of cells, relatively little is known about the mechanism by which overexpression establishes and maintains a malignant phenotype. In preliminary studies we found alterations in cell adhesion in a HER2-overexpressing cell line, suggesting that integrins, especially $\alpha_v\beta_3$, play a role in mediating cell adhesion in tumor cells. Our working hypothesis is as follows:

Hypothesis: HER2 overexpression causes changes in cell adhesion through formation of a multimeric complex that includes the integrin receptor $\alpha_v\beta_3$ and focal adhesion kinase (FAK). This causes disaggregation of integrin-mediated focal adhesions and disassociation from extracellular matrix proteins. A critical step in this process is proposed to be dephosphorylation of FAK by an as yet unidentified protein tyrosine phosphatase that interacts directly with HER2.

In order to address this hypothesis we proposed accomplishing a series of tasks. Our progress with these tasks is summarized below.

Task 1. Characterize changes in cell adhesion associated with HER2 overexpression using extracellular matrix proteins

We have used three paired cell lines (parental cell line and cell line stably transfected with HER2) to study the effect of HER2 overexpression on adhesion of cells to extracellular matrix proteins. The paired cell lines include the mouse fibroblast cell lines NIH-3T3/NIH-189, and human breast cancer cell lines MCF-7/MCF-7-HER2 and MDA-MB-468/MDA-MB-468-HER2. The MCF-7-HER2 cells were engineered in our laboratory. Figure 1 shows the amount of HER2 in each cell line compared to SKBR3 cells (a breast cancer cell line with constitutive overexpression of HER2).



cells are rounded and loosely attached to the culture vessel. There were no significant morphological changes associated with overexpression of HER2 in MCF7 cells (data not shown).

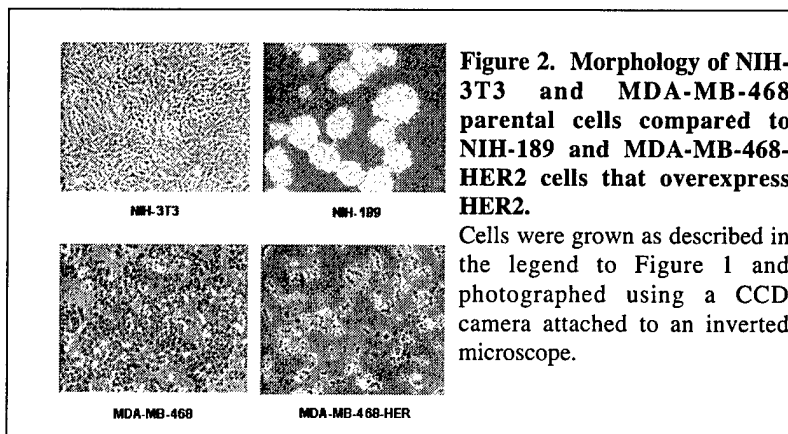


Figure 2 shows that overexpression of HER2 caused a dramatic change in the morphology of cells seeded on plastic tissue culture dishes. Whereas NIH-3T3 cells grow as an adherent monolayer, NIH-189 cells form clusters and exhibit anchorage-independent growth. Compared to MDA-MB-468 cells, MDA-MB-468-HER2

For adhesion assays, each cell line was grown in tissue culture medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were starved of serum overnight, dissociated with Versene/EDTA and plated in 48- or 96-well plates coated with human extracellular matrix proteins (fibronectin, vitronectin, native collagen

type I, heat-denatured collagen type I, collagen IV, and laminin) or poly-L-lysine. Poly-L-lysine coated wells were used as a control for non-integrin-mediated cell adhesion. Cells were incubated in growth medium without serum or antibiotics for the times indicated in the figures. Wells were washed three times with ice-cold PBS and stained with crystal violet. Crystal violet was quantified by colorimetric analysis at 600 nm after elution with 10% acetic acid.

Differences were observed in cell adhesion to ECM proteins in all paired cell lines. Compared with parental control NIH-3T3 cells, the HER2-overexpressing NIH-189 cells showed a reduction in cellular adhesion to each ECM protein (Figure 3A). HER2 overexpression also reduced the adhesion of MDA-MB-468 cells to each extracellular matrix protein (Figure 3B). Although the percentage reduction in cell adhesion of MDA-MB-468-HER2 cells to extracellular matrix proteins is less than that obtained with NIH-189 cells, the results are qualitatively similar suggesting a common mechanism for altered cell adhesion due to overexpression of HER2 in these cell lines. Interestingly, overexpression of HER2 in MCF-7 cells increased cell adhesion to extracellular matrix proteins (Figure 3C).

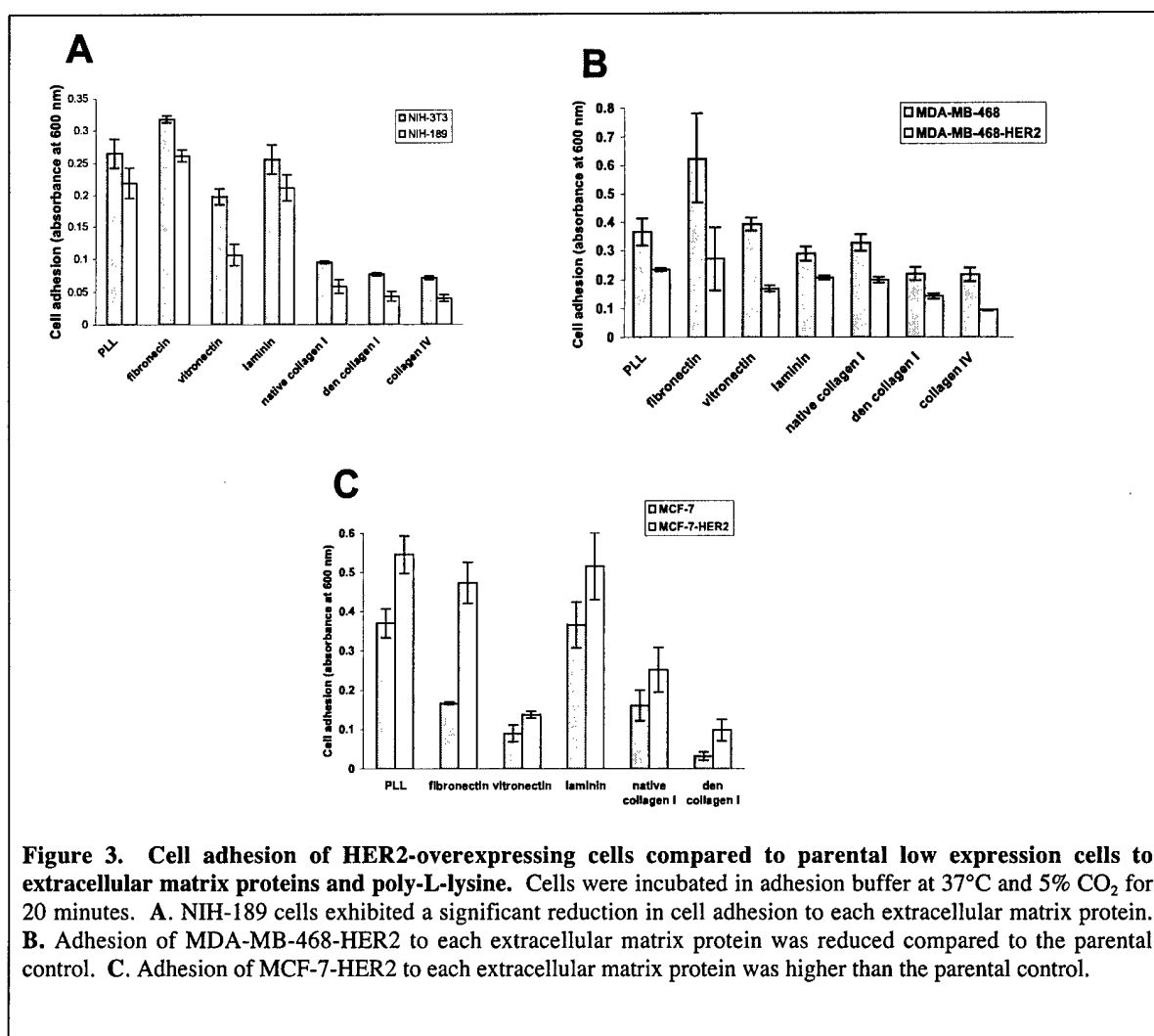
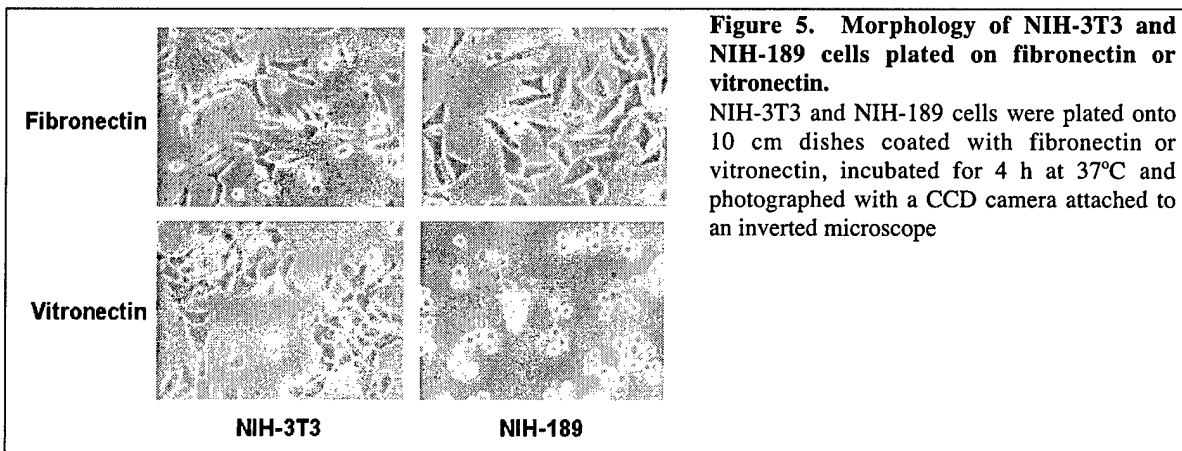
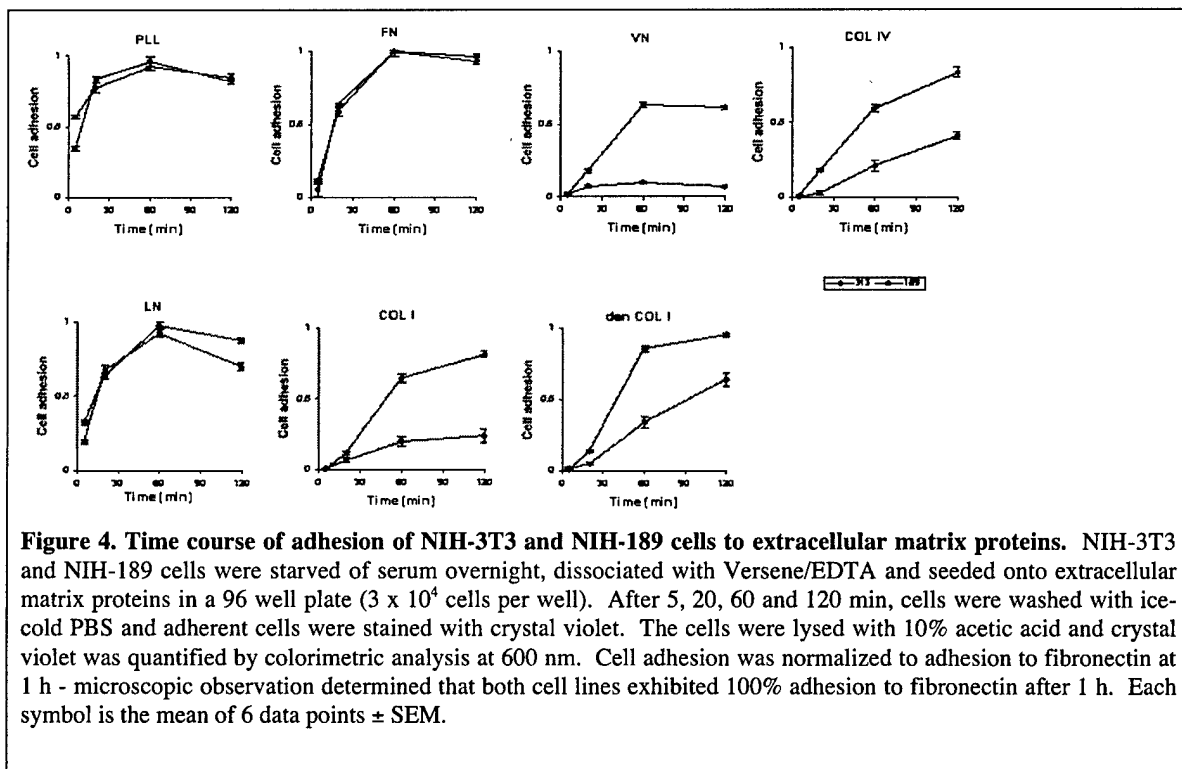


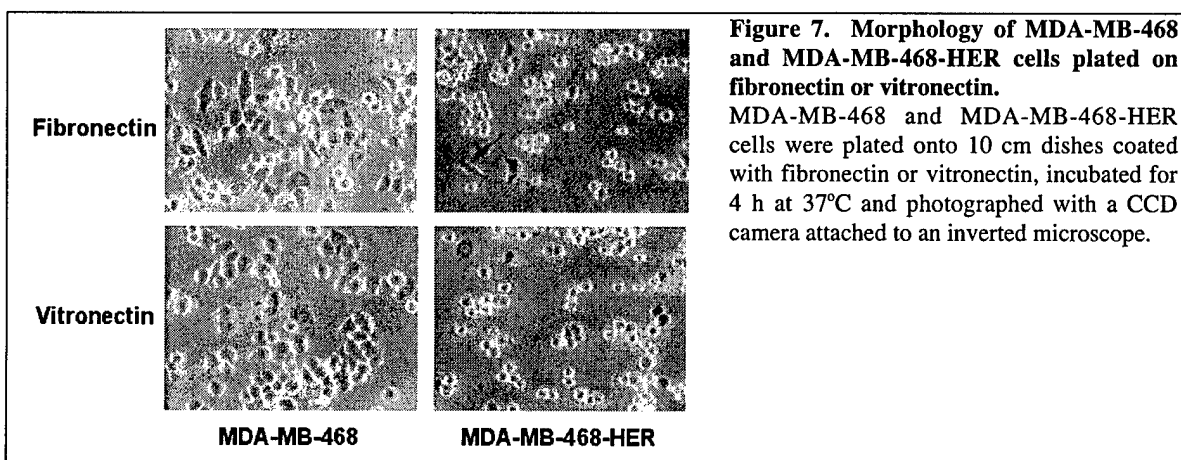
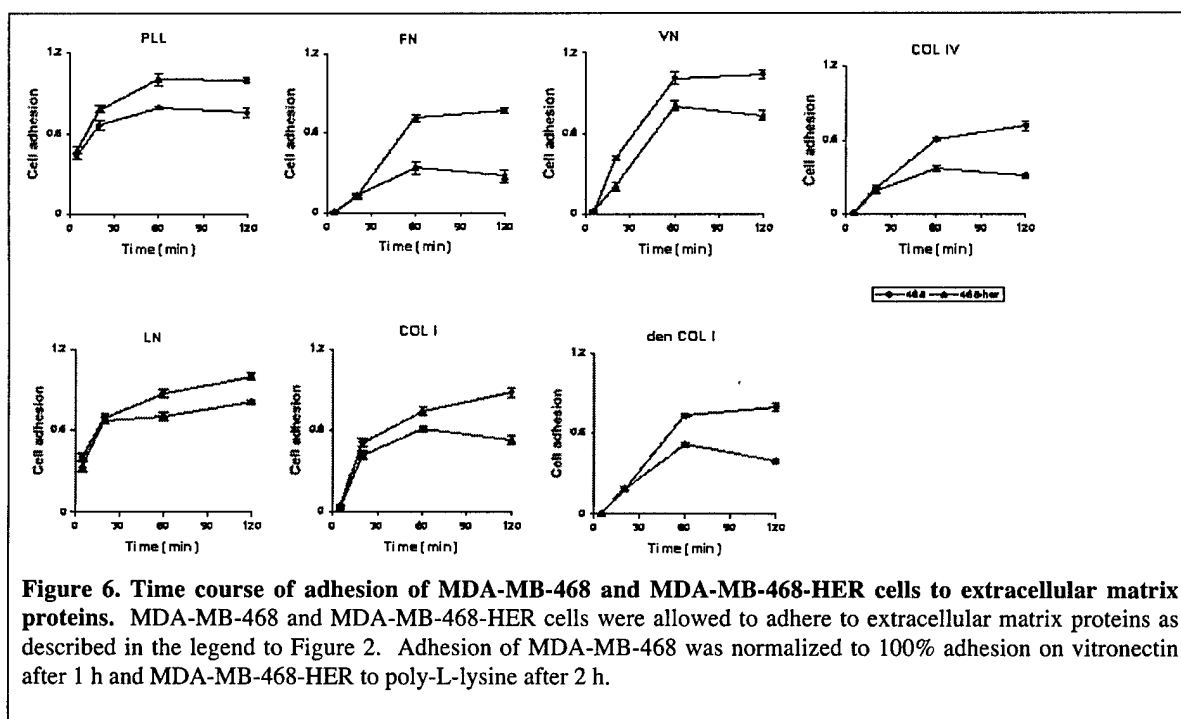
Figure 3. Cell adhesion of HER2-overexpressing cells compared to parental low expression cells to extracellular matrix proteins and poly-L-lysine. Cells were incubated in adhesion buffer at 37°C and 5% CO₂ for 20 minutes. **A.** NIH-189 cells exhibited a significant reduction in cell adhesion to each extracellular matrix protein. **B.** Adhesion of MDA-MB-468-HER2 to each extracellular matrix protein was reduced compared to the parental control. **C.** Adhesion of MCF-7-HER2 to each extracellular matrix protein was higher than the parental control.

We investigated the kinetics of cell adhesion between 5 min and 2 h in the NIH-3T3/NIH-189 and MDA-MB-468/MDA-MB-468-HER2 paired cell lines. Figure 4 shows that compared to NIH-3T3 cells, NIH-189 cells show a marked reduction in adhesion to vitronectin, collagen type IV, native collagen type I and denatured collagen type I between 20 min and 2 h. Overexpression of HER2 caused a reduction in adhesion to laminin after 2 h but had no significant effect on adhesion to fibronectin or poly-L-lysine. After 4 h on fibronectin both NIH-3T3 and NIH-189 cells are attached and spread and have similar morphological characteristics (Figure 5, upper panels). After 4 h on vitronectin NIH-189 cells remain rounded, are very loosely attached to the plate and begin to cluster together whereas NIH-3T3 cells are attached and spread (Figure 5, lower panels).



MDA-MB-468-HER2 cells showed reduced cell adhesion to each extracellular matrix protein (Figure 6) with only partial cell spreading after 4 h on fibronectin or vitronectin (Figure 7).

The results of the adhesion assays show that HER2 overexpression affects cell adhesion in a cell line- and extracellular matrix-dependent manner. It is interesting that the effect of altered cell adhesion is not a generalized effect: cell adhesion is reduced in NIH-189 and MDA-MB-468-HER2 cells and increased in MCF-7-HER2 cells.



In breast cancer cell lines and human tumors, overexpressed HER2 is recruited into heterodimers with other members of the HER family (EGFR, HER3 and HER4) that have been activated by epidermal growth factor (EGF) ligands. Dimerization induces receptor tyrosine kinase activity and autophosphorylation of specific phosphotyrosine residues (1) that leads to the stimulation of a number of signaling pathways (2, 3). A ligand for HER2 has not been identified and the role of HER2 is to act as a coreceptor. HER2 can function as a homodimer and is also the preferred partner for the other ligand-activated receptors, with the ligand-bound HER2/HER3 heterodimer demonstrating the most potent signaling (4-6). NIH-189 cells express low levels of EGFR and HER3 (data not shown). If HER2 is interacting with EGFR in NIH-189 cells we might expect

adhesion to be altered in the presence of growth factors: Lu et al (7) showed that in cells that overexpress EGFR, EGF treatment induced detachment of cells from the ECM. Figure 8 shows that HRG and EGF have no significant effect on adhesion of NIH-189 cells to fibronectin, vitronectin or poly-L-lysine. This suggests to us that overexpressed human HER2 can not heterodimerize with mouse EGFR or HER3 in NIH-189 cells and probably signals through HER2 homodimers.

We also investigated the effect of the HER2 inhibitor Herceptin on cell adhesion. Herceptin is a humanized monoclonal antibody that binds to the extracellular domain of HER2. The mechanism of action of Herceptin is complex. A number of mechanisms have been identified to date including antibody-dependent cellular toxicity, receptor downregulation from the cell surface, stimulation of HER2 homodimerization and partial prevention of heterodimer formation, inhibition of downstream signaling and inhibition of HER2 cleavage (8).

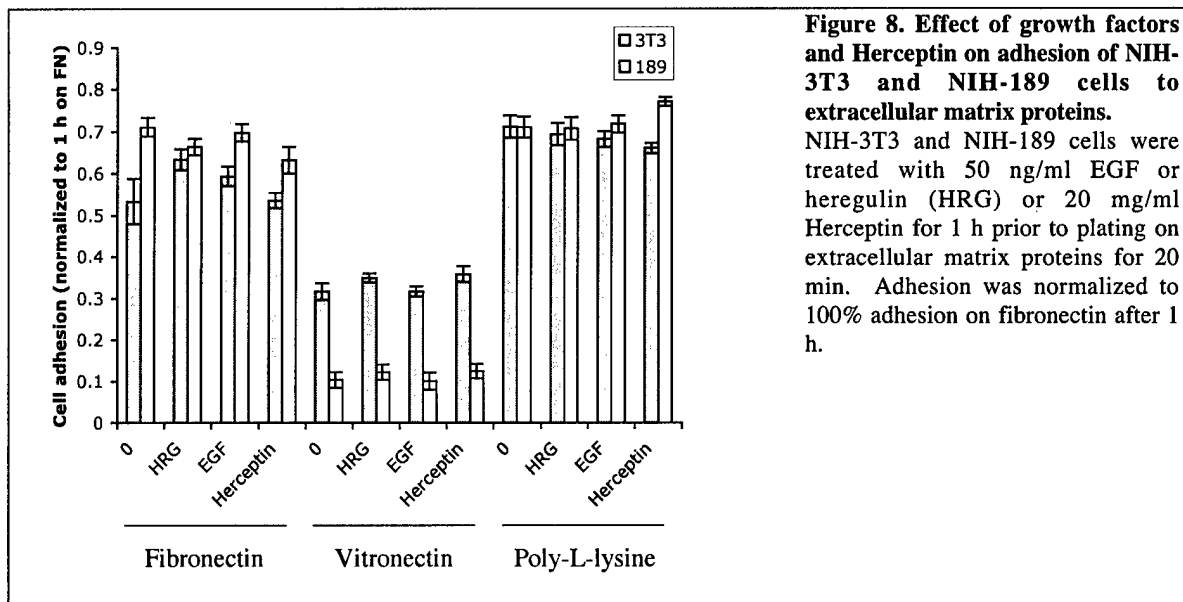


Figure 8. Effect of growth factors and Herceptin on adhesion of NIH-3T3 and NIH-189 cells to extracellular matrix proteins.

NIH-3T3 and NIH-189 cells were treated with 50 ng/ml EGF or heregulin (HRG) or 20 mg/ml Herceptin for 1 h prior to plating on extracellular matrix proteins for 20 min. Adhesion was normalized to 100% adhesion on fibronectin after 1 h.

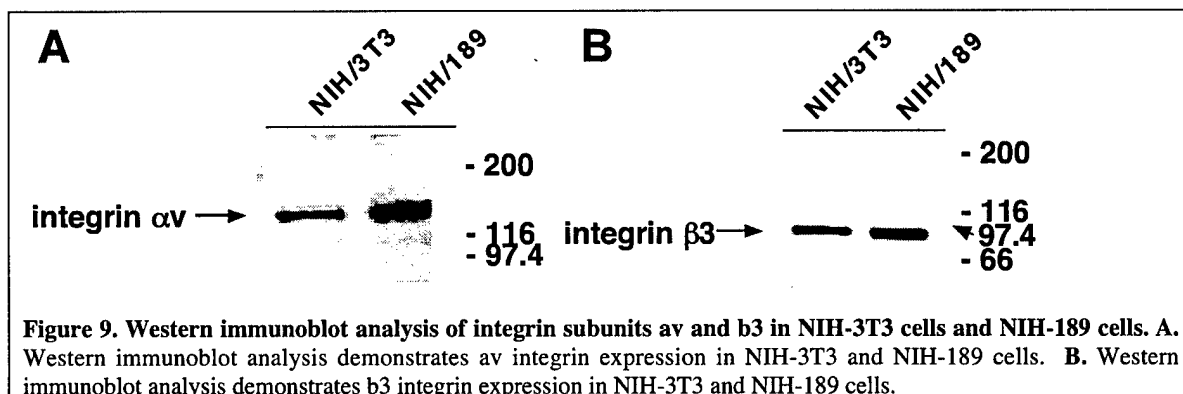
Figure 8 shows that Herceptin had no significant effect on cell adhesion. At this time we are unable to explain the inability of Herceptin to alter cell adhesion.

Potential molecular mechanisms responsible for changes in cell adhesion due to HER2 overexpression are discussed below.

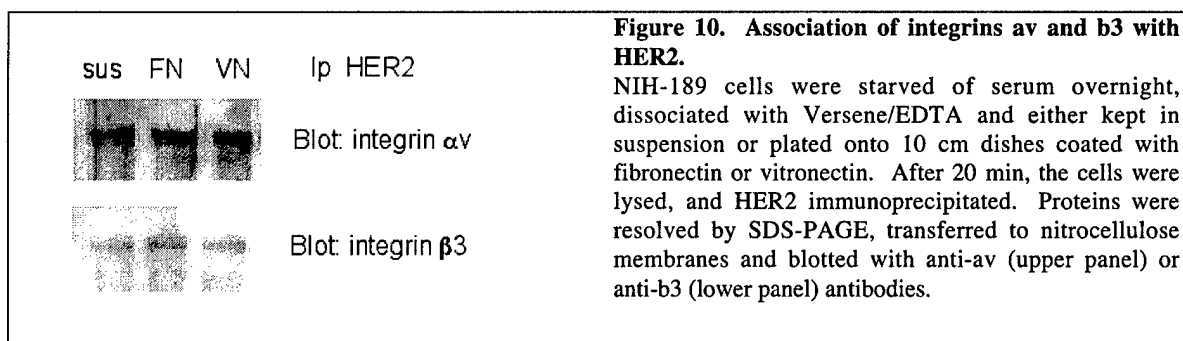
Task 2. Determine which integrins, if any, play a role in alterations of cell adhesion observed with HER2 overexpression

Our preliminary observations of differences in cell adhesion to various ECM proteins, described in Task 1, suggest that HER2 probably alters cell adhesion by interfering with integrin-mediated binding to extracellular matrix proteins. In HER2-overexpressing NIH-189 cells, vitronectin showed the most impressive alteration in adhesion. Vitronectin is associated with binding by the $\alpha v \beta 3$ integrin receptor. Although other integrins ($\alpha v \beta 1$ and $\alpha v \beta 5$) can bind vitronectin, the $\alpha v \beta 3$ integrin is known as the "vitronectin receptor". In addition, the $\alpha v \beta 3$ integrin receptor is largely responsible for adhesion to denatured collagen, type I. Because the $\alpha v \beta 3$ integrin receptor appeared to be a pivotal molecule in mediating at least some of the HER2-associated alterations

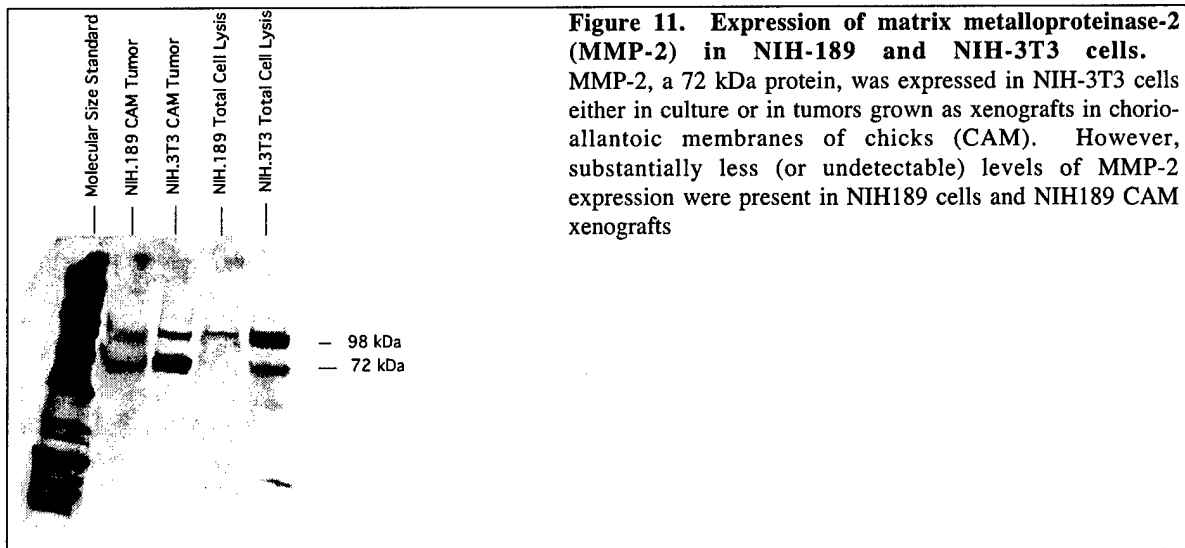
in cell adhesion in NIH-189 cells, we assessed expression levels of this integrin. The simplest explanation for reduced adhesion to vitronectin would be a reduction in expression of avb3 integrin in HER2-overexpressing cells compared with parental cells. We performed western immunoblot analysis in order to test this hypothesis and were surprised to find no reduction in either av or b3 subunits in HER2-overexpressing NIH-189 cells compared to NIH-3T3 cells (Figure 9).



Moro *et al* (9), showed that during the early phase of cell adhesion the EGFR transiently associates with avb3: the complex disassembles 5 minutes after integrin engagement. Also, EGFR did not form a complex with avb3 when the cells were kept in suspension. To investigate whether HER2 forms a complex with avb3, NIH-189 cells were starved of serum overnight and either kept in suspension or plated onto dishes coated with fibronectin or vitronectin for 20 min. The cells were lysed, immunoprecipitated with HER2 antibody and blotted with antibodies to integrin αv or $\beta 3$. Figure 10 shows that integrins αv and $\beta 3$ are associated with HER2 in cells kept in suspension or plated on fibronectin or vitronectin for 20 min.



We believe that HER2 overexpression may reduce adhesion of NIH-189 cells to vitronectin by preventing disassembly of the avb3 /HER2 complex by an unknown mechanism. Another potential source of altered cell adhesion mediated by avb3 could be co-expression of matrix metalloproteinase type 2 (MMP-2) with binding of MMP-2 at the carboxy-terminal PEX domain to the avb3 integrin receptor (10).



Denatured collagen is observed in tissues during proteolytic digestion of the ECM in angiogenesis and in tumor cell invasion (10, 11). ECM degradation requires the coordinated interaction of matrix metalloproteinases - especially MMP-2 - and integrins. Since MMP-2 is bound to $\alpha v \beta 3$ integrin during ECM degradation, $\alpha v \beta 3$ could be important in other aspects of tumor growth in addition to cell adhesion (10). This possibility was investigated with western immunoblot analysis of cells grown in culture and tumors grown as xenografts in the chorio-allantoic membranes of chick embryos (10, 11) (Figure 11). Interestingly, MMP-2 was expressed by NIH-3T3 cells grown in culture and in chorio-allantoic membranes but was not expressed by HER2-overexpressing NIH-189 cells in culture or in chorio-allantoic membranes (Figure 10). A monoclonal antibody, TV88, to the carboxyterminal PEX domain was also used in order to exclude the possibility that the PEX domain alone was associated with $\alpha v \beta 3$ integrin after activation of MMP-2 with cleavage at the PEX domain (data not shown). This was the opposite of what would have been predicted if MMP-2 or PEX associated with $\alpha v \beta 3$ to alter cell adhesion. As a result of these observations we considered the possibility that HER2 overexpression affected alterations in cell adhesion through alterations in the signal transduction pathways of the cell.

Task 3. Assess the molecular mechanism responsible for alterations in cell adhesion of HER2 - over expressing cells .

Our working hypothesis was that activation of FAK, a key, signaling molecule regulating integrin-mediated adhesion, is altered by HER2 overexpression. FAK contains a number of sites of tyrosine phosphorylation including tyrosine 397, the major site of autophosphorylation, and tyrosine 577 within the catalytic domain (12).

NIH-3T3 and NIH-189 were either kept in suspension or plated on fibronectin or vitronectin for 20 min. Cell extracts were immunoprecipitated with FAK antibody and blotted with an antibody to phosphotyrosine. Figure 12 shows that there was a slight increase in total phosphorylation of FAK when cells were plated on ECM protein. However, there was no effect of HER2 overexpression on total FAK phosphorylation.

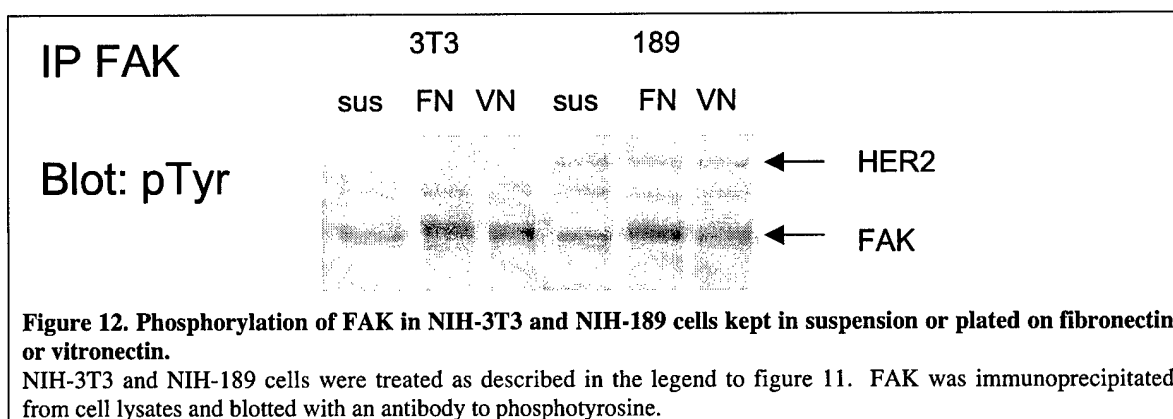
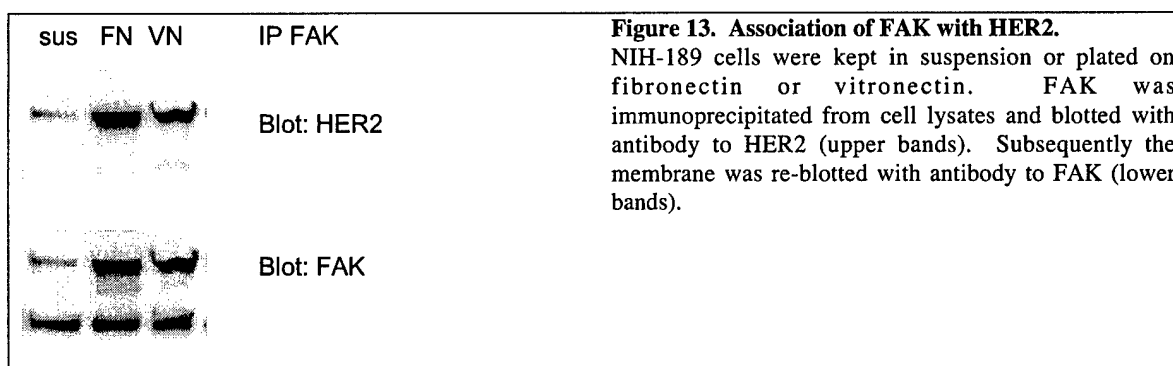
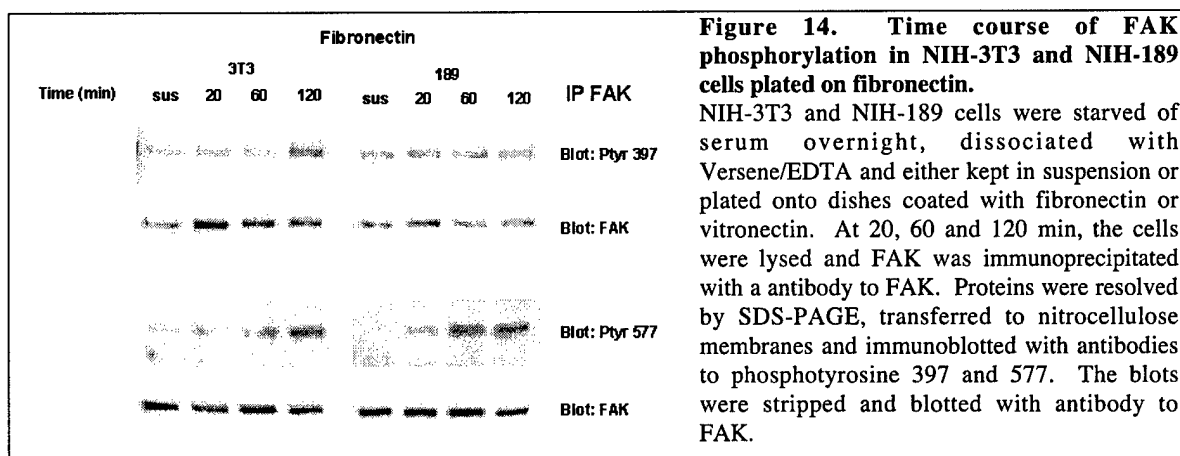


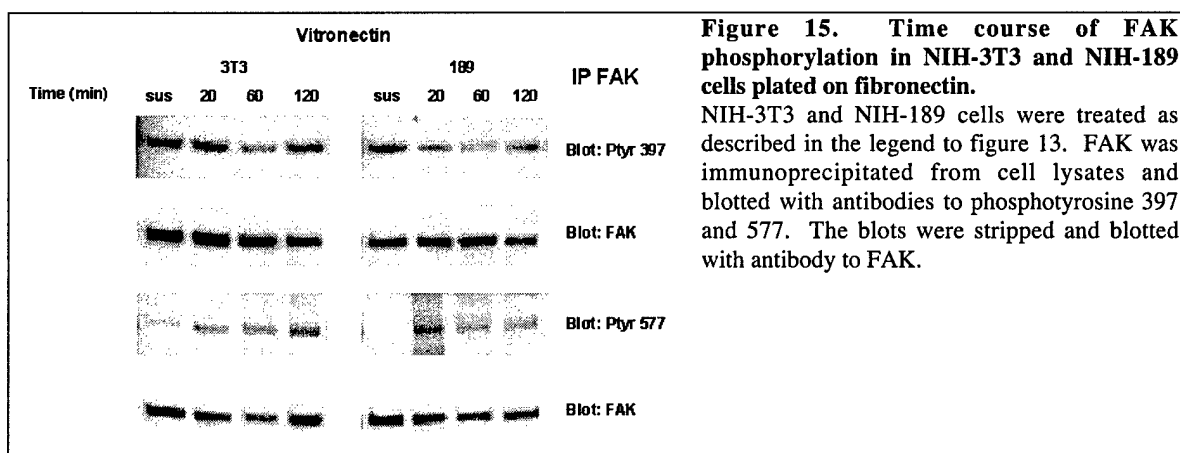
Figure 12 also shows that, in NIH-189 cells, FAK is associated with a 180 kDa, tyrosine phosphorylated protein, which is the size of HER2. This association was confirmed by immunoprecipitating FAK from cell lysates and sequentially blotting for HER2 and FAK (Figure 13). The association between FAK and HER2 was stronger when NIH-189 cells were plated on ECMs.



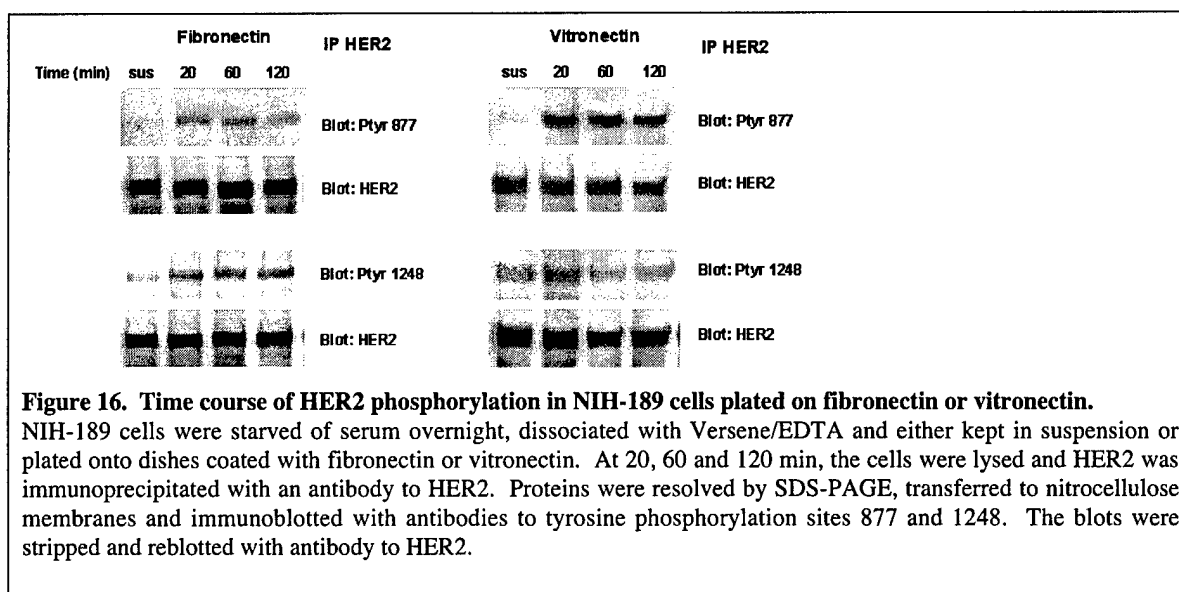
Although there was no difference in total FAK phosphorylation between NIH-3T3 and NIH-189 cells we determined the levels of phosphorylation at tyrosines 397 and 577 since reduction of phosphorylation at one or more of these sites may be masked by phosphorylation at other tyrosine residues. Phosphorylation of FAK at tyrosine 397 remained constant and did not depend on the extracellular matrix protein or HER2 overexpression (Figure 14 and 15, upper panels). Phosphorylation at tyrosine 577 was low when the cells were kept in suspension and increased when the cells were plated on fibronectin and vitronectin (Figure 14 and 15, lower panels).



Since NIH-3T3 and NIH-189 cells show identical kinetics of adhesion to fibronectin, we expected similar levels of FAK phosphorylation in the two cell lines. However, we did not expect levels of phosphorylation to be the same in both cell lines exposed to vitronectin. Thus, a reduction in cell adhesion due to overexpression of HER2 does not appear to be due to changes in the phosphorylation of FAK at tyrosines 397 and 577. However, we can not rule out changes in phosphorylation at other phosphorylation sites on FAK.



It is well documented that integrins regulate the function of receptor protein tyrosine kinases (13, 14). When cells expressing EGFR adhere to extracellular matrix proteins, EGFR is rapidly phosphorylated and remains phosphorylated for at least 30 min (9, 14, 15). NIH-189 cells plated onto fibronectin or vitronectin exhibited phosphorylation of HER2 at tyrosine 877 that remained constant for 2 h after cell adhesion (Figure 16, upper panels). Phosphotyrosine 877 is located within the kinase domain of HER2 and phosphorylation at this site is important for the regulation of the kinase function of HER2 (16). Interestingly, phosphorylation at tyrosine 877 was higher when cells were plated on vitronectin even though cells adhere poorly to this extracellular matrix protein.



Phosphotyrosine 1248 is the major autophosphorylation site for HER2 (17). There was no change in phosphorylation at this site when the cells were plated on fibronectin or vitronectin (Figure 16, lower panels). Thus, changes in cell adhesion caused by overexpression of HER2 are not due to extracellular matrix protein-dependent changes in HER2 phosphorylation.

Cell adhesion is a two-step process that involves cell attachment and cell spreading. Although NIH-189 cells exhibit reduced adhesion to vitronectin, the cells are loosely attached to this ECM (Task 1, Figure 5). Moro *et al* (15) showed that EGFR undergoes integrin-mediated phosphorylation in the absence of cell spreading. We believe that attachment of NIH-189 cells to vitronectin is sufficient to induce HER2 phosphorylation.

Since FAK is primed for cell adhesion and spreading on vitronectin, we conclude that other molecules involved in cell adhesion are altered by HER2 overexpression.

Key Research Accomplishments

The research accomplishments described in the Body of this Progress Report can be summarized as follows:

1. We have determined the effect of HER2 overexpression on cell adhesion in three paired cell lines. The reduction in cell adhesion due to HER2 overexpression is dependent on the cell line and the extracellular matrix protein.
2. Cell adhesion in HER2-overexpressing NIH-189 cells is not affected by growth factors, or the HER2-specific inhibitor Herceptin.
3. In NIH-189 cells, integrins α_v and β_3 remain associated with HER2 when the cells are plated on ECMs.

4. FAK is phosphorylated when cells are plated on fibronectin and vitronectin and the level of phosphorylation is the same in NIH-3T3 and NIH-189 cells.
5. In NIH-189 cells FAK associates with HER2 when the cells are kept in suspension or plated on ECMs.
6. HER2 is phosphorylated when cells are plated on vitronectin and fibronectin. Phosphorylation at tyrosine 877 increases when the cells are exposed to vitronectin and fibronectin. Phosphorylation at tyrosine 1248 is not dependent on the ECM.

Reportable Outcomes

Cell line

Engineered human breast cancer cell line MCF-7 that overexpresses human HER2.

Abstract

Mechanism for loss of cell adhesion in HER-2/neu overexpressing tumor cells. Michael F. Press, Jinha Park, Kathryn Boorer, Dorothy Hong, Isabell Schmitt, and Lucia Pham. 2002, Era of Hope, Orlando, Florida.

Conclusions

Overexpression of HER2 is associated with alterations in cell adhesion to specific extracellular matrix proteins, implicating integrin receptors in mediating this process. Alterations in cell adhesion due to overexpression of HER2 are cell line-dependent. The observed differences in adhesion between cell lines may be due to the expression levels of the other HER family members. For example, NIH-3T3 cells express low levels of EGFR, HER2 and HER3; MCF7 cells express EGFR, HER2 and HER3 but do not show overexpression of any of these receptors (18); MDA-MB-468 cells show amplification and overexpression of the EGFR, but have low level expression of HER2 and of HER3 (18, 19).

Changes in cell adhesion due to overexpression of HER2 are not due to changes in FAK or HER2 phosphorylation.

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